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Identification of the atrial natriuretic factor-R_{1C} receptor subtype (B-clone) in cultured rat aortic smooth muscle cells

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The present report demonstrates the presence in cultured rat aortic smooth muscle cells of a natriuretic factor receptor subtype with a specificity typical of the ANF-R_{1C} (B-clone) receptor subtype. To prove the existence of this receptor subtype in this cell line we show that pCNP-(82–103) is the most potent activator of the intrinsic guanylate cyclase activity, and that [^{125}I]pCNP-(82–103) binds to a specific receptor subtype which is insensitive to the ANF-R_2 specific ligand, C-ANF. The investigation of its binding characteristics show the rank potency order of the natriuretic factors in competing for pCNP binding to be pCNP > pBNP > rANF. Furthermore it was possible to covalently photolabel this receptor subtype with underivatized [^{125}I]pCNP and show that it is composed of a single subunit of 130 kDa with very high specificity for pCNP.

Atrial natriuretic factor; Brain natriuretic peptide; C-Type natriuretic peptide; Guanylate cyclase; Natriuretic factor receptor

1. INTRODUCTION

Signal transduction of natriuretic factors involves the binding to specific high affinity membrane receptors. These atrial natriuretic factors (ANF) receptors are divided into two main classes: ANF-R_1 and ANF-R_2 receptors. The first class, ANF-R_1, which possesses a guanylate cyclase catalytic activity, is subdivided into several subtypes with distinct pharmacological specificity for the different natriuretic factors [1,2]. Two of these have been cloned, the ANF-R_{1A} subtype (A-clone) and the ANF-R_{1C} subtype (B-clone). The ANF-R_{1A} subtype is mostly specific for both ANF and brain natriuretic peptide (BNP) [3,4] and is found at high concentration in many target organs of these natriuretic peptides [5]. The ANF-R_{1C} subtype is mainly specific for C-type natriuretic peptide (CNP) [2] and its distribution is limited to tissues of neuronal origin [5]. The ANF-R_{1B} subtype, which has not yet been cloned, displays higher affinity for ANF than for BNP [1] and does not recognize CNP. The ANF-R_2 receptor class, which has been cloned, does not discriminate between these peptides [6], is widely distributed [5] and recognizes biologically inactive ANF derivatives e.g. [Cys^{116}]rANF-(102–116)×-NH_2 (C-ANF). Until recently there was no direct evidence showing the existence of the ANF-R_{1C} receptor subtype specific for CNP binding. A report showing that CNP was more potent than ANF in increasing cGMP production in cultured rat aortic smooth muscle cells (RASM) [7] indicated that this subtype might be present in a cultured non-neuronal cell type. Although it is known from studies using recombinant receptor that the ANF-R_{1C} is specific for CNP, no reports have shown a complete characterization of this receptor subtype in target organs for natriuretic factors. In order to prove the pharmacological relevance of the ANF-R_{1C} subtype, it is of prime importance to identify and characterize this receptor subtype in such target organs. Our results show a complete characterization of the binding and enzymatic properties of this receptor subtype in cultured rat aortic smooth muscle cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Rat aortic smooth muscle cells (RASM) were a gift from Dr. P. Hamet of the Research Center of the Hotel-Dieu Hospital of Montreal. They were grown in Dulbecco's modified Eagle medium supplemented with 5% calf serum and 100 U of penicillin–streptomycin (Gibco, Canada).

2.2. *Cyclic GMP determination*

Upon reaching confluence cell monolayers were stimulated in Dulbecco's modified Eagle medium in the presence of 0.5 mM IBMX as previously described [6] and extracellular cyclic GMP production was measured by radioimmunoassay [6].

2.3. *Preparation of membranes*

Confluent RASM cells were washed 3 times with ice-cold saline and scrapped from their plates. After a 10 min centrifugation at 250 × *g* the cell pellet was homogenized in 10 vols. of buffer A (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM EDTA, 100 mM NaCl, 10^{-7} M aprotinin, 10^{-6} M leupeptin, and 10^{-7} M pepstatin A) with a polytron homogenizer. The homogenate was centrifuged at 600 × *g* for 10 min, the pellet discarded, and the supernatant was then centrifuged at 100,000 × *g* for 30 min. After a second centrifugation the resulting pellet was resuspended in buffer B (50 mM Tris-HCl, 0.1 mM EDTA, 250 mM sucrose, 1 mM $MgCl_2$, pH 7.4). The membranes were frozen in liquid nitrogen and stored at −70°C until used.

2.4. *Iodination procedure*

Tyr^0-pCNP-(82–103) (Peninsula Lab., CA), and succinimidyl-cyclic GMP-tyrosyl-methyl-ester (Sigma Chemicals, MO) were iodinated with the enzymobead technique (Bio-Rad) according to the directives of the manufacturer. The mono-iodinated product was purified on RP-HPLC as described [8].

2.5. *Receptor binding assay*

The binding studies were carried out essentially as previously described [9] in the presence or absence of C-ANF (0.1 μM) at 4°C overnight.

2.6. *Photoaffinity labeling protocol*

Membranes from RASM cells were photoaffinity labeled as previously reported [10]. Briefly, 80 μg/ml of membrane proteins were incubated in the dark at 10°C for 18 h with 30 pM of [^{125}I]Tyr^0-pCNP in the presence or absence of unlabeled peptides in 1 ml of buffer C (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.5% BSA, and 5 mM $MnCl_2$). After the incubation period the membranes were photolysed for 20 min. Following irradiation the membrane proteins were pelleted at 12,000 × g for 15 min, washed once in buffer C without BSA, resuspended in 100 μl of sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% Bromophenol blue, and 5% 2-mercaptoethanol) and heated at 100°C for 3 min prior to electrophoresis.

2.7. *SDS-polyacrylamide gel electrophoresis*

Proteins were analyzed by electrophoresis on a 7.5% acrylamide gel according to the method of Laemmli [11]. An amount of 80 μg of proteins was loaded onto each lane. Autoradiograms are shown after 8–10 days exposure of the fixed-dried gels at −70°C.

2.8. *Data analysis*

Dose–response curves for cyclic GMP production were analyzed with the four-parameter logistic equation [12]. Competition binding curves were analyzed with a non-linear least-squares curve fitting program to obtain estimates of the binding equilibrium constant of the different peptides [13].

3. RESULTS

Characterization of the natriuretic peptide pharmacological profile in rat aortic smooth muscle cells shows that pCNP is the most potent stimulator of the intrinsic guanylate cyclase activity of the receptor protein, both

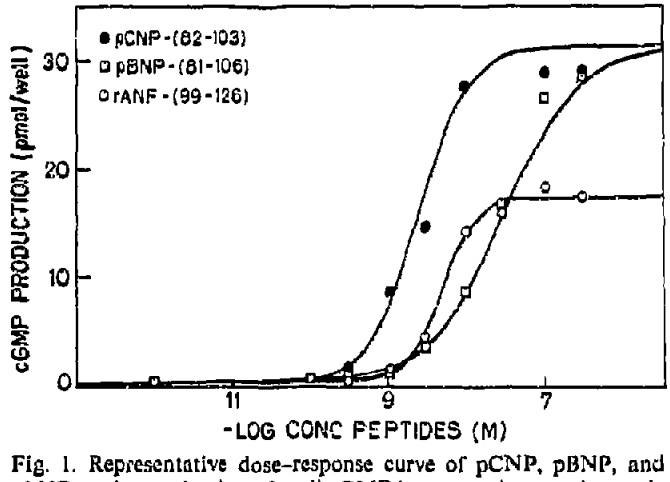

Fig. 1. Representative dose–response curve of pCNP, pBNP, and rANF on the production of cyclic GMP by rat aortic smooth muscle cells in culture. The cells were challenged with the different peptides for 3 h at 37°C and extracellular cyclic GMP was assayed as described [6].

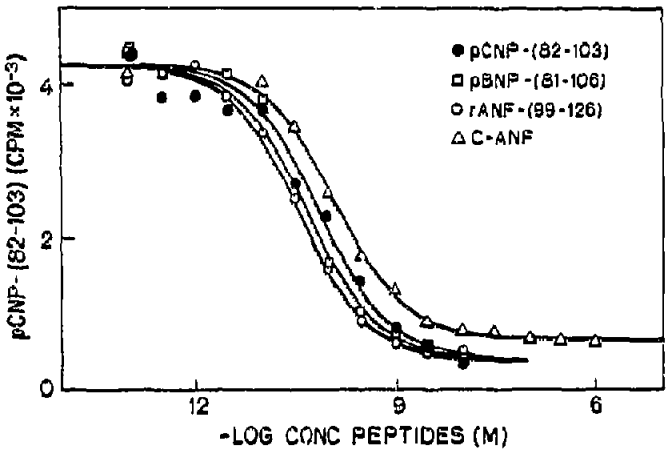

Fig. 2. Competition binding curves of different natriuretic factors for the binding of [^{125}I]pCNP-(82–103) to RASM cell membranes in the absence of C-ANF.

in terms of ED_{50} and maximal effect (Fig. 1). pCNP stimulates the enzyme up to 450-fold with an ED_{50} of 5 nM, while rANF and pBNP were less potent with ED_{50}'s of 9.5 and 27.6 nM respectively. When the enzyme activity was assayed directly on RASM cell membranes pCNP was still the most potent activator of guanylate cyclase activity (data not shown). In addition we have observed that the ANF-R_2 specific derivative, C-ANF, did not antagonize the stimulatory effect of either pCNP, ANF or BNP in RASM cells (data not shown).

The binding specificity of the natriuretic peptide receptor present on RASM cell membranes was also in-

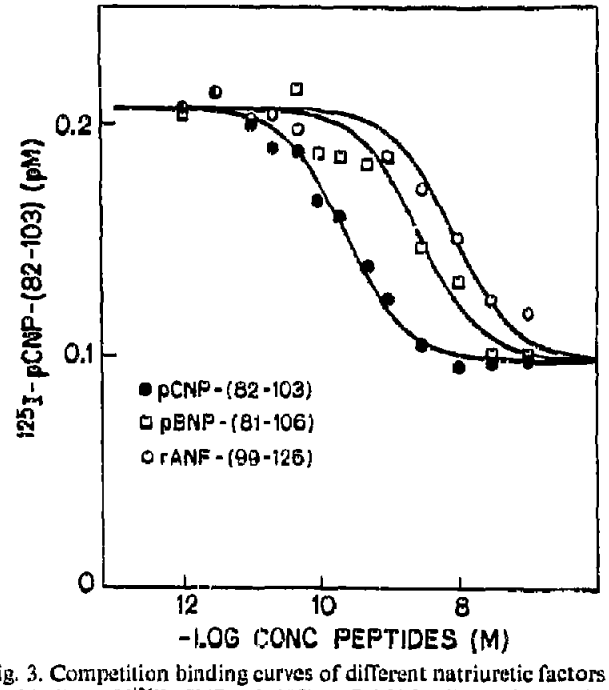

Fig. 3. Competition binding curves of different natriuretic factors for the binding of [^{125}I]pCNP-(82–103) to RASM cell membranes in the presence of C-ANF (0.1 μM).

vestigated. Fig. 2 illustrates that [^{125}I]Tyr^0-pCNP-(82–103) labels a population of receptor sites with a specificity indicative of a predominant ANF-R_2 class and a total density of sites of 490 fmol/mg of protein. The majority of receptors present on RASM cell membranes do not discriminate between the different natriuretic peptides as they recognize all the natriuretic peptides with similar affinities. Fig. 2 also shows that C-ANF potently competed for 90% of [^{125}I]pCNP-(82–103) specific binding sites.

In order to investigate the C-ANF insensitive sites we have characterized their pharmacological binding profile in the presence of a saturating concentration of C-ANF in order to block the ANF-R_2 sites. Fig. 3 shows that the rank potency order for this receptor subtype is: pCNP > pBNP > rANF, with a selectivity ratio for pCNP over rANF of 50-fold and over pBNP of 13-fold, and a density of 60 fmol/mg of protein. This CNP-specific receptor was photoaffinity labeled with [^{125}I]Tyr^0-pCNP-(82–103) in order to determine its molecular size. Fig. 4 shows the specific photolabeling of 2 protein bands: a minor band at 130 kDa, corresponding to the pCNP-specific ANF-R_{1C} subtype, and a major band at 64 kDa, corresponding to the ANF-R_2 subtype. The selectivity of the receptor band was assessed by competition of [^{125}I]Tyr^0-pCNP-(82–103) labeling with increasing concentrations of unlabeled pCNP or rANF in the presence of a saturating concentration of C-ANF. Fig. 5 clearly demonstrates that the 130-kDa high molecular weight protein is specific for pCNP and has a lower affinity for rANF, and corresponds to the ANF-R_{1C} subtype documented in Fig. 3.

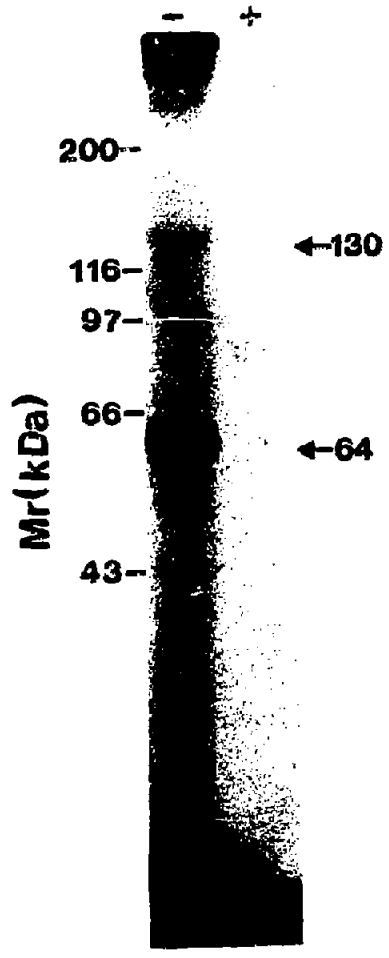

Fig. 4. Photoaffinity labeling of the ANF-R_{1C} receptor subtype by [^{125}I]pCNP in RASM cell membrane in the presence or absence of unlabeled pCNP. Membrane proteins were subjected to SDS-gel electrophoresis on a 7.5% acrylamide gel. Molecular weight protein standards (Da) are myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,699), and carbonic anhydrase (31,000).

4. DISCUSSION

The characterization of the binding profile of the ANF-R_{1C} receptor subtype in RASM cells is the same as that obtained from the expressed receptor clones [2]

Fig. 5. Competition of the labeling of the ANF-R_{1C} by [^{125}I]pCNP with increasing concentrations of unlabeled pCNP and rANF.

i.e. pCNP > pBNP > rANF, indicating that we have effectively identified the same ANF-R_{1C} (B-clone) receptor subtype that was previously revealed by guanylate cyclase studies [7]. Examination of the CNP binding characteristics of the major ANF receptor population in RASM, in the absence of C-ANF, reveals a profile that is typical of ANF-R_2 receptors. But interestingly blockade by C-ANF of ANF-R_2 sites discloses the ANF-R_{1C} sites corresponding to about 10% of the total density of CNP-specific binding sites and which was not documented in previous studies. In addition to these functional aspects of the ANF-R_{1C} receptor we have analyzed its protein structure after photoaffinity labeling with [^{125}I]pCNP. Like the ANF-R_{1A} the ANF-R_{1C} is a 130-kDa monomer. The specificity of the photolabeled receptor protein was demonstrated by direct competition of [^{125}I]pCNP-(82–103) binding with pCNP and rANF, showing that this receptor subtype was specific for pCNP and had very low affinity for rANF.

These results contrast somewhat with previous reports which showed that pCNP could only label a 70-kDa protein band in cultured rat aortic smooth muscle cells, corresponding to the ANF-R_2 receptor subtype [7]. It is possible that this 70-kDa band might have resulted from proteolytic cleavage of a higher molecular weight protein band as previously reported [14]. In our study proteolytic attack of the ANF-R_{1C} receptor subtype was avoided by introducing protease inhibitors. The observation of a lower maximal stimulation of the enzymatic activity produced by rANF, in contrast to pBNP and pCNP, is still unclear. It is possible that this cell type expresses very low levels of ANF-R_{1A} receptor subtype, which preferentially responds to rANF, therefore explaining the lower levels of cGMP attained with rANF. Alternatively the fact that rANF does not show a higher potency, which would be consistent with its high affinity on the ANF-R_{1A} receptor subtype, is possibly due to its phosphorylation by an ecto-cAMP-dependent protein kinase [15], which would be responsible for its lower potency consistent with its decreased vasorelaxant activity [16]: however, the presence of such ectokinase in RASM cells remains to be proven. We could also suggest that this difference in maximal cGMP production might be due to a lower efficacy of rANF on the ANF-R_{1C} receptor subtype and that the lower potency of rANF is only the resultant potency of the peptide on a majority of ANF-R_{1C} and a minority of ANF-R_{1A} receptor subtypes in RASM cells.

In summary we have identified and characterized the ANF-R_{1C} (B-clone) receptor subtype in cultured rat aortic smooth muscle cells. We show that it is specific for pCNP and has a very low affinity for rANF, which shows a very low efficacy on the activation of this receptor subtype's enzymatic activity. This receptor subtype is present in small amounts and represents 10–15% of the total binding capacity for natriuretic peptides in this cell type, the rest being contributed by the ANF-R_2 subtype.

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